AD_____

Award Number: DAMD17-01-1-0574

TITLE: Demonstration that a mRNA Binding Protein is Responsible for GADD45 mRNA Destabilization

PRINCIPAL INVESTIGATOR: Steve F. Abcouwer, Ph.D.

CONTRACTING ORGANIZATION: University of New Mexico Health Sciences Center
Albuquerque, New Mexico 87131-5041

REPORT DATE: May 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Final (1 May 2002 - 30 Apr 2003)

4. TITLE AND SUBTITLE
Demonstration that a mRNA Binding Protein is Responsible for GADD45 mRNA Destabilization

5. FUNDING NUMBERS
DAMD17-01-1-0574

6. AUTHOR(S)
Steve F. Abcouwer, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of New Mexico Health Sciences Center
Albuquerque, New Mexico 87131-5041
E-Mail: sabcouwer@salud.um.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We are studying the post-transcriptional control of expression of the p53-inducible antiproliferative gene known as Growth Arrest and DNA Damage induced gene 45 (GADD45). Using regions of the GADD45 mRNA 3'-untranslated region (UTR) in RNA gel shift assays, we have observed that glutamine causes distinct changes in RBP activities in cytoplasmic and nuclear protein extracts. RNA-affinity columns were used to enrich extracts for RBPs binding to the distal region of GADD45 3'-UTR. Eluded proteins were subjected to Western blotting, to detect specific RBPs, as well as proteomic analysis. Data suggest that the distal region of the GADD45 3'-UTR is bound by the destabilizing RBP known as AU-binding factor 1 (AUF1). Western blotting demonstrated that AUF1 is present in extracts from both glutamine-fed and glutamine-starved cells. However, when cytoplasmic extracts from glutamine-starved cells are subjected to RNA-affinity purification, the AUF1 does not bind to the distal GADD45 3'-UTR region. These results suggest that the effect of glutamine deprivation on GADD45 mRNA stability is orchestrated by AUF1 binding, either through inhibition of AUF1 RNA binding activity or through competitive inhibition by another RBP. Regions of GADD45 mRNA are now being tested for the ability to confer glutamine-mediated instability to fusion mRNAs.

14. SUBJECT TERMS

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

We are examining the molecular mechanism by which the expression of an antiproliferative p53 downstream effector gene is post-transcriptionally controlled in breast cancer cells. The downstream effector gene to be studied is the growth arrest and DNA damage induced gene, GADD45. This gene is transcriptionally activated by wild-type p53; therefore GADD45 expression can be depressed in p53-deficient cells. Employing GADD45 knockout mice, A. J. Fornace and colleagues found that loss of GADD45 expression reproduced a large subset of the effects observed in p53 knockout mice (1). It is theorized that increasing GADD45 expression in p53-deficient cells will reproduce many of p53's antiproliferative functions. GADD45 expression in breast carcinoma cell lines is tightly controlled by the availability of the amino acid glutamine, primarily through a post-transcriptional mechanism (2). GADD45 mRNA is inherently unstable with a half-life of 30 to 45 minutes. Depriving these cell of media glutamine increased the half-life of GADD45 mRNA by approximately 17-fold. Conversely, repletion of media glutamine caused an immediate and rapid decay of GADD45 mRNA. Thus, this model system can be used to determine the mechanism by which GADD45 gene expression is controlled through mRNA turnover. In analogy to destabilization of AU-rich mRNAs such as c-myc by the AU-rich binding factor AUF1 (3), it is hypothesized that there exists a mRNA binding protein that binds to GADD45 and causes or initiates its degradation. Our experiments have implicated AUF1 in the control of GADD45 mRNA binding. We have found that AUF1 can bind to the extreme 3' region of GADD45 mRNA and that the ability of AUF1 to bind to this region is affected by glutamine availability. We are currently testing the ability of this region of GADD45 mRNA (as well as other regions) to confer glutamine-dependent instability to a heterologous mRNA. We are also determining the identity of other proteins that bind to the extreme 3' region of GADD45 mRNA. These experiments are expected to increase our knowledge of how the expression of this downstream effector of p53 action is controlled and may suggest a means to post-transcriptionally increase GADD45 expression and thus inhibit the growth of p53-defficient tumors.

BODY

Using human breast carcinoma cell lines, we have demonstrated that the half-life of GADD45 mRNA is very responsive to ambient glutamine (GLN) availability. It is hypothesized that specific RNA binding proteins function in a GLN-dependent fashion to orchestrate GADD45 mRNA decay. This could involve an RNA binding protein (RBP) that is activated by GLN and causes GADD45 mRNA decay, or an RBP that is activated by GLN deprivation and blocks the degradation of GADD45 mRNA. In the second scenario, the stabilizing factor would be expected to displace or block the action of a constitutive destabilizing RBP (Figure 1). By comparing RNA binding activities in cytoplasmic lysates from TSE breast carcinoma cells cultured in the presence of GLN and cells subjected to GLN deprivation the existence of a GLN-responsive RPB is being tested. Using regions of the GADD45 mRNA 3' untranslated region (UTR) in RNA gel shift assays (Figure 2), we have observed that glutamine causes distinct changes in RBP activities in cytoplasmic and nuclear lysates (Figure 3).

RNA affinity columns were used to enrich extracts for RBPs binding to the distal region of GADD45 3' UTR. Eluded proteins have been subjected to Western blotting, to detect specific

RBP, as well as proteomic analysis. Preliminary data suggest that the distal region of the GADD45 3' UTR is bound by the destabilizing RBP known as AU-binding factor 1 (AUF1). Western blotting demonstrated that AUF1 is present in lysates from both glutamine-fed and glutamine-starved cells. However, when lysates from glutamine-starved cells are subjected to RNA affinity purification, the AUF1 does not bind to the distal GADD45 3'-UTR region (Figure 4). These results suggest that the effect of glutamine on GADD45 mRNA stability could be orchestrated by AUF1 binding, either through inhibition of AUF1 RNA binding activity or through competitive inhibition by another RBP. Recently, we have also submitted gel-fractionated proteins eluded from the RNA affinity columns for MALDI-TOF analysis by the UNM Proteomics Core Facility. It is hoped that this analysis will help to identify additional RBPs that are both binding to GADD45 mRNA under all conditions and those that are differentially binding in response to glutamine deprivation (Figure 2).

Although no longer supported, work on this project will continue until a publishable conclusion is reached. Chimeric GADD45-CAT cDNA constructs have now been constructed (Figure 2), but have not yet been used in an experiment. We will transfect these into breast carcinoma cells and assay the effects of glutamine on the turnover of the chimeric mRNAs transcribed from these constructs. We expect that these experiments will show that fusion of the distal 3'-UTR fragment of GADD45 mRNA to the CAT mRNA will confer glutamine-dependent stability on the chimeric mRNA. We will then seek to confirm the effects of AUF1 binding on GADD45 stability by blocking AUF1 expression using RNA silencing. We will also examine the effects of glutamine deprivation on AUF1 post-translational modifications, namely phosphorylation.

Personnel paid salary from this grant include the PI, Steve F. Abcouwer, Assistant Professor, Yann Klimentidis, a part-time student employee laboratory technician, and Olena Barbash, a Ph.D. candidate who joined the lab in January. Olena Barbash is now being partially supported by the UNM Department of Biochemistry and Molecular Biology so that she can continue this research project, at least to the point where the work is published.

KEY RESEARCH ACCOMPLISHMENTS:

- Constructed several GADD45 cDNA-containing plasmid vectors.
- Evaluated several methods to transfect TSE cells.
- Optimized transfection by cationic lipid method.
- Optimized transfection by electroporation method.
- Perfected RNA gel shift assays methods
- Used RNA gel shifts to examine differences in GADD45 RNA binding protein (RBP) activities in cell extracts from glutamine-fed and glutamine-starved cells (Figure 3).
- Determined that a major difference in RBP activity was caused by glutamine deprivation (Figure 3). This was the major hypothesis to be tested. Thus, our major hypothesis has been proven.
- Determined that a major differential RBP activity is targeted to the distal 3' region of GADD45 mRNA sequence (Figure 3).
- Developed RNA-affinity column method of enriching GADD45 RBPs.
- Used RNA affinity column containing a distal 3' region of GADD45 mRNA (bases 1051-1325) to enrich RBPs from extracts of glutamine-fed and glutamine-starved cells (Figure 4).

- Used Western blotting to examine presence of HuR and AUF1 proteins in enriched RBP fractions. Found that AUF1 protein was bound to GADD45 distal 5' region only when present in lysates from glutamine-fed cells, even though AUF1 protein was present in lysates from glutamine-starved cells (Figure 4).
- Have PCR cloned several sections of GADD45 mRNA and inserted into a vector containing chloramphenicol acetyl transferase (CAT) cDNA to create chimeric fusion cDNAs to be used for testing the effect of GADD45 mRNA regions on glutamine-dependent mRNA stability (figure 2).
- A portion of the work was presented in poster form by Olena Barbash, a Ph.D. candidate, at the Biomedical Sciences Graduate Program (BSGP) Research Day Poster Presentation and Contest.
- An abstract of this work was submitted to the Federation of Associated Societies for Experimental Biology (FASEB) Summer Research Conference entitled "Control of Gene Expression by Dietary Constituents." This conference included scientific sessions on control of gene expression by amino acids as well as on post-transcriptional control of gene expression by nutrients.

REPORTABLE OUTCOMES:

- GADD45 cDNA plasmid constructs
- GADD45-CAT chimeric fusion cDNA constructs (Figure 2)
- GADD45 mRNA affinity columns (Figure 4)
- Demonstration that glutamine does affect GADD45 RBP activity (Figure 3)
- Demonstration that AUF1 binds to distal 5'-UTR sequences (1051-1325) of GADD45 mRNA (Figure 4).
- Demonstration that AUF1 protein present in cytoplasmic lysates from glutamine-starved cells does not bind to GADD45 distal 5'-UTR sequences (Figure 4).
- Poster presented at BSGP Research Day Poster Presentation and Contest
- Abstract submitted to FASEB Summer Research Conference

CONCLUSIONS:

The major hypothesis to be tested by this concept award grant-funded project has been validated. The work has allowed us to demonstrate that glutamine deprivation does affect the activity of RBPs that bind to GADD45 mRNA. This is the first data to suggest a mechanism by which expression of this antiproliferative gene is controlled on a post-transcriptional level. We have identified one RBP, AUF1, as a factor that binds to GADD45 mRNA in a glutamine-dependent fashion. Given that this is a know destabilization factor, it is likely that AUF1 binding to GADD45 mRNA causes it to be unstable under glutamine-fed conditions, and that the lack of AUF1 binding under glutamine-starved conditions contributes to the observed gain of stability. We have also identified the distal 3'-UTR region of GADD45 mRNA (bases 1051-1325) as an AUF1-binding region. We have also developed and/or produced all the tools needed to test if this region (or another region) of GADD45 mRNA is sufficient and necessary to confer glutamine-dependent instability on a heterologous mRNA. We have also enriched, gel purified and

processed several proteins that bind to the distal GADD45 3'-UTR region and have submitted these samples for proteomic analysis by MALTOF. This analysis will hopefully identify additional RBPs that bind to this mRNA fragment, some of which exhibit glutamine-dependent binding. Although a publication has not yet resulted from this work, we are planning to present the work in poster form this summer. We will continue the work until a publication is obtained will seek new sources of internal and external funding to support the project.

REFERENCES

1. Hollander, M.C. et al. Disruption of gadd45 leads to genomic instability, loss of cellular growth control and radiation-induced carcinogenesis. Proc Amer Assoc Cancer Res 40:413 [abstract #2728], 1999.

2. S.F. Abcouwer, C. Schwarz and R. A. Mequid. Glutamine deprivation induces the expression of GADD45 and GADD153 primarily by mRNA stabilization. J Biol Chem 247:28645-28651, 1999.

3. Wilson, G.M. and G. Brewer. The search for trans-acting factors controlling messenger RNA decay. Prog. Nucleic Acid Res Mol Biol 62:257-91, 1999.

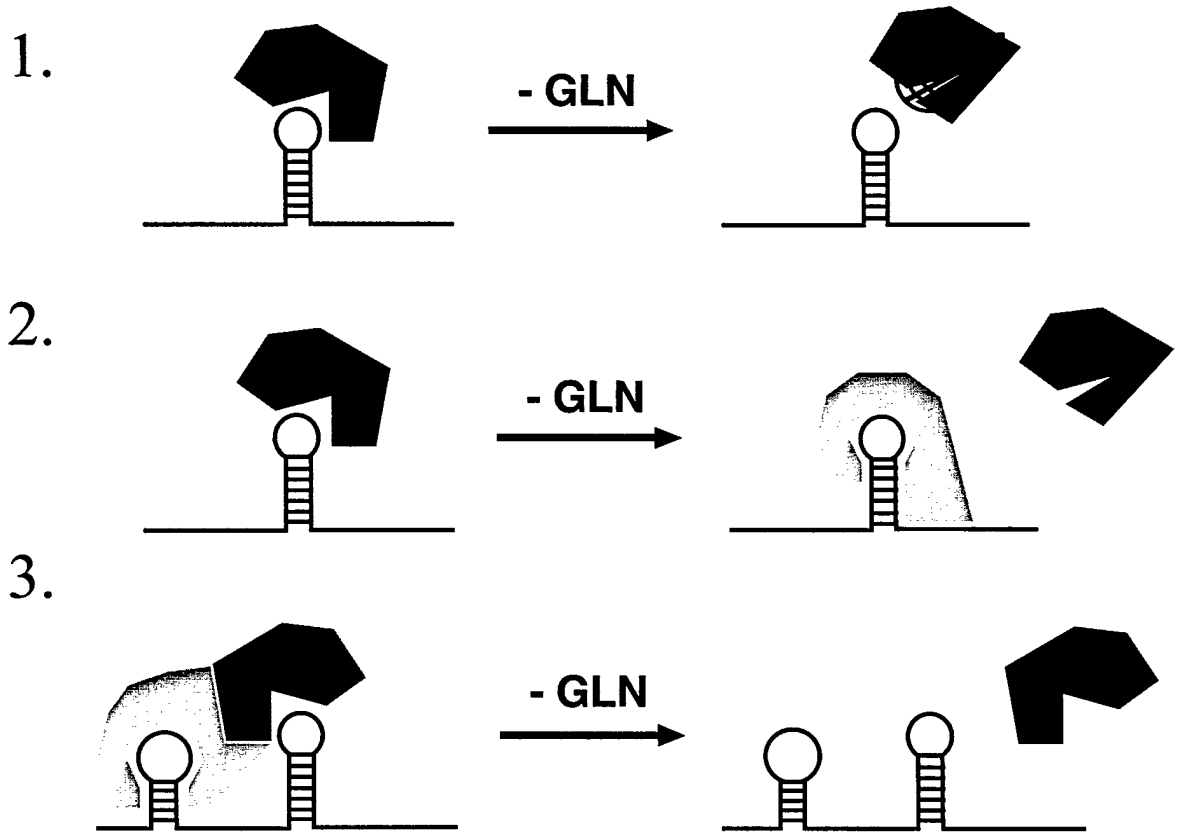

Fig 1. Proposed mechanisms for the regulation of GADD45 mRNA stability. It is hypothesized that specific RNA binding proteins function in a GLN-dependent fashion to orchestrate GADD45 mRNA decay. Three possible mechanisms are proposed: 1) This could involve an RNA binding protein (RBP) that is activated by GLN and causes GADD45 mRNA decay. In the presence of GLN this protein looses its ability to bind to GADD45 mRNA. 2) In the second scenario, the stabilizing factor would be expected to displace or block the action of a constitutive destabilizing RBP. 3) Destabilizing mRNA-binding protein may be recruited to mRNA sequence by binding to some cofactor in the presence of GLN ("blind" binding). GLN deprivation causes the whole complex to dissociate from RNA, promoting GADD45 message stabilization.

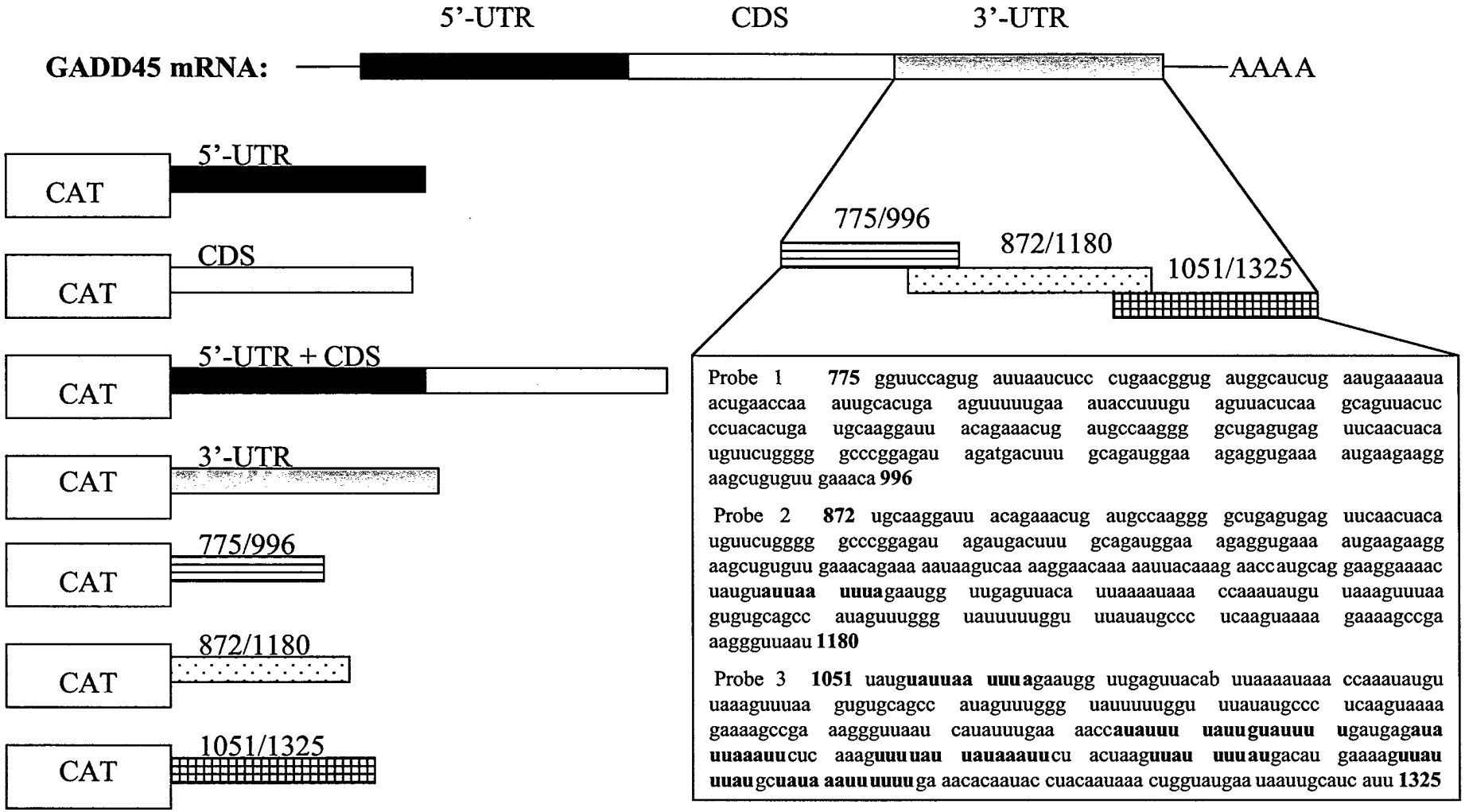

Figure 2. GADD45 mRNA and ARE sequences used for contruction of GADD45-CAT chimeric cDNA contructs and used for RNA probes in gel shift experiments. Fragments corresponding to 5'-UTR, CDS, 5"-UTR + CDS, 3'-UTR and 3'UTR subfragments 775/996, 872/1180, 1051/1325 (Genbank accession# NM_015675). For use as probes, these fragments were obtained by PCR amplification. For construction of chimeric cDNAs for mRNA stability analysis, these fragments were PCR amplified using modified primers with inserted KpnI and BamHI restriction sites. After restriction digestion, sequences were ligated into pcDNA3/CAT vector (Invitrogen) following the CAT cDNA, to created chimeric fusion cDNAs.

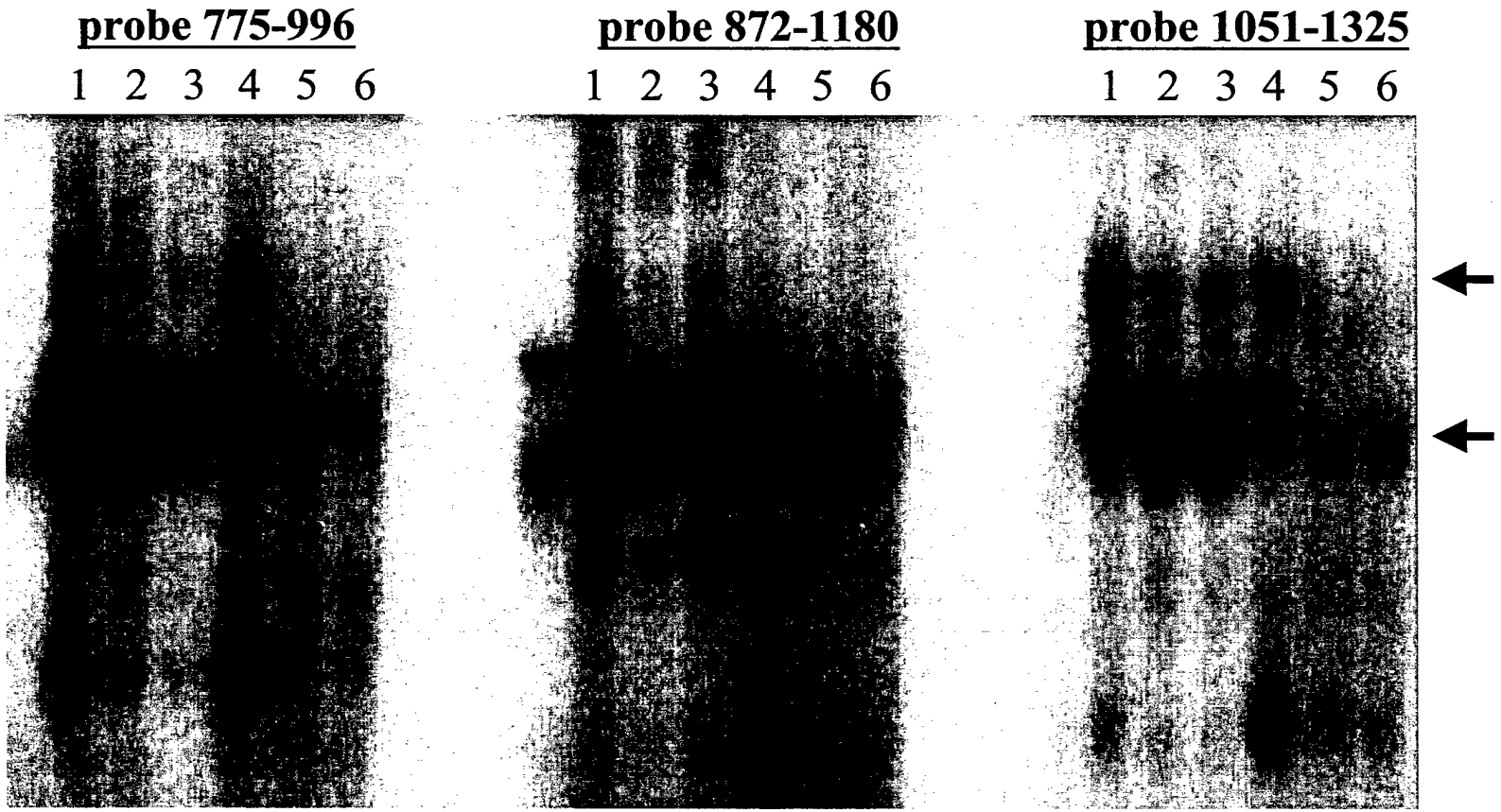

Fig 3. Glutamine deprivation causes distinct changes in RBPs activities in cytoplasmic and nuclear extracts. Subconfluent TSE cells were starved for glutamine for 19 hours and then fed with glutamine-free medium to which 0 or 4 mM GLN was added. Cells were harvested after 2 h treatment and nuclear and cytoplasmic extracts isolated using TransFactor kit (Clontech). RNA probes corresponding to GADD45 3'UTR fragments from 775 through 996, 872-1180 and 1051-1325 were generated using in vitro transcription with radioactive ATP. RNA probes were incubated with indicated protein extracts, and formed complexes resolved on 5% TBE PAGE gel. Lanes 1, 2 and 3 are shifts with cytoplasmic extracts and lanes 4, 5 and 6 are shifts with nuclear extracts. Extracts were obtained from (1and 4) glutamine fed controls, (2 and 5) glutamine starved cells, and (3 and 6) cells starved and then refed with glutamine.Among several differences caused by glutamine starvation is the pronounced absence of one complex (black arrow) and increased presence of another complex (gray arrow) formed with probe 1051-1325.

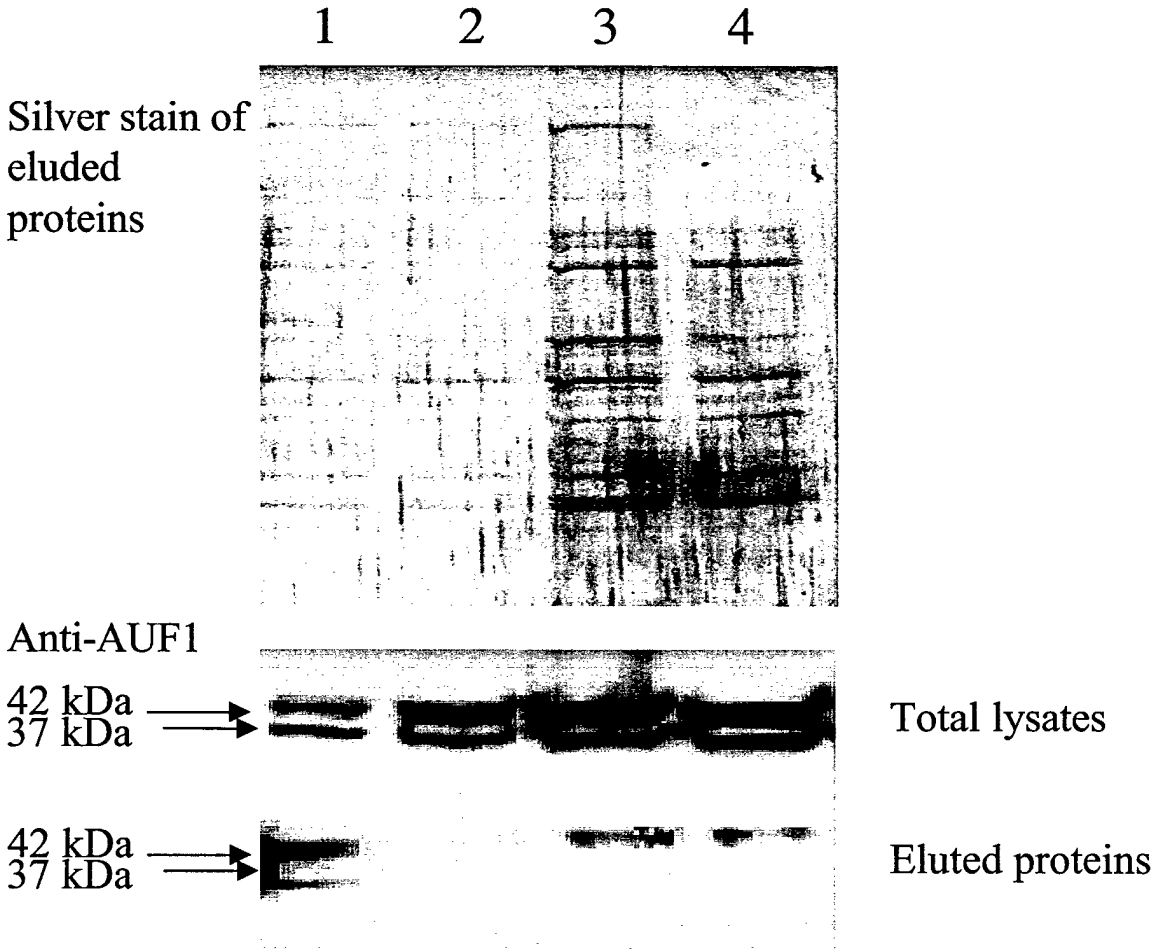

Fig 4. GLN deprivation changes repertoire of proteins that bind to the 3'UTR of GADD45 mRNA. Non-radioactive GADD45 mRNA probe 1051-1325 was attached to streptavidin-coated magnetic beads using corresponding biotinylated complimentary oligos (IDT). Cytoplasmic and nuclear lysates from glutamine fed and glutamine starved cells loaded onto collumns. After washing away unbound proteins, proteins bound to RNA column were eluted and separated on SDS-PAGE gels. Western blotting was performed using rabbit polyclonal anti-AUF1 sera. Lanes 1 and 2 represent eluded proteins from cytoplasmic extracts from glutmine-fed and glutamine-starved cells, respectively. Lanes 3 and 4 represent eluded proteins from nuclear extracts lysates from glutmine-fed and glutamine-starved cells, respectively. Note that AUF1 protein was present in all extracts, but AUF1 in cytoplasmic extracts from glutamine-starved cells did not bind to the column.